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A Water-Soluble Luminescence Oxygen Sensor

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Abstract

We developed a water-soluble luminescent probe for dissolved oxygen. This probe is based on $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$, which is a sulfonated analogue of the well-known oxygen probe $(\text{Ru}[\text{dpp}]_3)\text{Cl}_2$. The compound dpp is 4,7-diphenyl-1,10-phenanthroline and $\text{dpp}(\text{SO}_3\text{Na})_2$ is a disulfonated derivative of the same ligand. In aqueous solution in the absence of oxygen $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ displays a lifetime of 3.7 μs that decreases to 930 ns on equilibrium with air and 227 ns on equilibrium with 100% oxygen. The Stern–Volmer quenching constant is 11330 M^{-1} . This high oxygen-quenching constant means that the photoluminescence of $\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3\text{Cl}_2$ is 10% quenched at an oxygen concentration of $8.8 \times 10^{-6}\text{ M}$, or equilibration with 5.4 torr of oxygen. The oxygen probe dissolved in water displays minimal interactions with lipid vesicles composed of dipalmitoyl-L- α -phosphatidyl glycerol but does appear to interact with human serum albumin. The absorption maximum near 480 nm, long lifetime and large Stokes' shift allow this probe to be used with simple instrumentation based on a light-emitting diode light source, allowing low-cost oxygen sensing in aqueous solutions. To the best of our knowledge this is the first practical water-soluble oxygen sensor.

INTRODUCTION

Quenching of fluorescence by oxygen is one of the earliest observations in fluorescence quenching (1). Since then, a variety of fluorophores with longer lifetimes have also been used as indicators of dissolved oxygen (2,3). Long-lifetime phosphorescent porphyrins have been developed for oxygen sensing (4). One of the most widely used oxygen sensors has been ruthenium-(4,7-diphenyl-1,10-phenanthroline) $_3$, $^{\dagger}(\text{Ru}[\text{dpp}]_3)^{2+}$. The favorable optical properties of this complex have resulted in considerable effort to improve the performance of oxygen sensors based on this luminescent metal–ligand complex (MLC) (6–9). Sensors based on ruthenium-tris-diphenylphenanthroline $(\text{Ru}[\text{dpp}_3])^{2+}$ are highly stable and can even be steam sterilized (10). To improve further the spectral properties of these oxygen sensors, MLC excitable with green light have been developed (11), as have oxygen sensors that can be excited above 600 nm (12,13). These long-wavelength oxygen sensors allow measurements through skin (12), suggesting the possibility of minimally invasive

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† Abbreviations: dpp, 4,7-diphenyl-1,10-phenanthroline; $\text{dpp}(\text{SO}_3\text{Na})_2$, 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt; DPPG, dipalmitoyl-L- α -phosphatidylglycerol; FD, frequency domain; FLIM, fluorescence lifetime imaging microscopy; HSA, human serum albumin; LED, light-emitting diode; MLC, metal–ligand complex.

transdermal sensing. The possibility of minimally invasive sensing is supported by the use of decay times instead of intensities (12,14,15) because decay times can be measured in turbid media and through skin (16).

In all the above reports, the fluorophores were insoluble in water and either dissolved in solvents or were contained in polymeric supports. While the use of silicon as a support has proven valuable for purposes of avoiding interferences (17), there are many instances where water-soluble sensors could be of value. For instance, water samples could be mixed with small amounts of a water-soluble sensor and the oxygen concentration determined from the intensity or preferably the decay time, which would be insensitive to the probe concentration. Because the sensor concentration would not be important, the sensor could be added using a simple dropper bottle. Another application of a water-soluble oxygen sensor would be imaging of intracellular oxygen concentrations. To facilitate such applications we developed the water-soluble ruthenium complex shown in Fig. 1. This compound was found to have similar spectral properties and oxygen sensitivity as the unsulfonated MLC and thus seems suitable as a water-soluble oxygen sensor.

MATERIALS AND METHODS

The RuCl_3 and 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt ($\text{dpp}[\text{SO}_3\text{Na}]_2$) were obtained from Aldrich and used without further purification. Sephadex LH-20 was purchased from Pharmacia. Human serum albumin (HSA) and DPPG (dipalmitoyl-L- α -phosphatidylglycerol) were obtained from Sigma and used without further purification. Solutions of HSA (5 mg/mL) in 0.1 M phosphate buffer were maintained at pH 7.2 for all experiments. Lipid vesicles were prepared by sonication of DPPG (2 mg/mL) in 10 mM Tris with 50 mM KCl at pH 7.5.

Oxygen Stern–Volmer quenching constants (K_{SV}) and bimolecular quenching constants were determined by varying the O_2 concentration in aqueous solutions containing $\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3\text{Cl}_2$. Oxygen (>99%) was taken from a cylinder and mixed with argon by using a Linde mass flow controller to yield gases of defined percent O_2 composition. The proper composition was prebubbled through 100 mL H_2O and then bubbled through the analyte solution. The precision of the gas-mixing device is specified to be within $\pm 1\%$. The concentration of O_2 in H_2O was determined by scaling the O_2 composition to that of the total solubility of O_2 in H_2O (0.00125 M at 100% O_2).

Synthesis of $\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3\text{Cl}_2 \cdot 6\text{H}_2\text{O}$.

This compound was synthesized as previously stated with slight modification (18). The RuCl_3 (57 mg, 0.0276 mmol) was reacted with 3.5 equivalents of 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt (518 mg, 0.0965 mmol) in 20 mL deionized H_2O and refluxed with stirring for 2 days. The solution changed from dark green to red over this time. The solution was cooled to room temperature and filtered. The solvent was removed by rotovap and the solid redissolved in 5 mL H_2O . This solution was loaded onto a Sephadex LH-20 column (25 $\times$ 2 cm) and eluted with H_2O . The first brown and purple bands were discarded and the red fraction collected. The red fraction was evaporated to dryness to give a red solid. Characterization was consistent with the literature (18). It should

be noted that this compound is a mixture of several isomers. These isomers differ because SO₃Na substituents on the phenyl rings are randomly positioned. However, there is no evidence for a heterogeneous mixture of long-lifetime probes as ascertained by frequency-domain (FD) fluorometry.

Electronic absorption spectra were measured on a Hewlett Packard HP-8453 spectrophotometer. Steady-state photoluminescence spectra were acquired on an SLM Aminco AB-2 spectrofluorometer with 480 ± 2 nm excitation. The optical density of (Ru[dpp(SO₃Na)₂]₃)Cl₂ was at or below 0.2 at 480 nm during all measurements. Time-resolved photoluminescence decays were measured in the FD with instrumentation that has been described previously (19). The excitation source was an air-cooled continuous-wave Ar⁺ laser (543-AP, Omnicrome) tuned to the 488.0 nm line. The laser was amplitude modulated with a low-frequency modulator (K2.LF, ISS) and input into an ISS FD fluorometer (Koala). Data were collected at ~20 distinct frequencies between 7 kHz and 2 MHz. Emission was collected through a 610 nm cutoff filter (Coming 2-61) and Texas Red with a lifetime of 4 ns was used as a lifetime reference.

The FD intensity decay data were fit to single- and multiexponential models. The analyses of these decays were performed with nonlinear least-squares procedures described previously (19). The intensity decays were described by

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

where α_i are the amplitudes of each component of the decay and τ_i are the respective decay times, and $\alpha_i = 1.0$.

RESULTS

Absorption and emission spectra of Ru(dpp[SO₃Na]₂)₃ when dissolved in water are shown in Fig. 2. The spectra are similar to that displayed by (Ru[dpp]₃)²⁺. The absorption near 480 nm allows excitation with blue light-emitting diodes (LED). The large Stokes' shift is a favorable property of this class of compounds because it allows for easy rejection of scattered light. Additionally, a large Stokes' shift typically means that the lumophores will not self-quench by probe-probe interactions.

Emission spectra of the water-soluble (Ru[dpp(SO₃Na)₂]₃)²⁺ are shown in Fig. 3. In the presence of dissolved oxygen from the air, the intensity decreases 2.9-fold, compared to that in the absence of oxygen. In the presence of dissolved oxygen equivalent to 100% O₂, the intensity decreases 16.4-fold, relative to the O₂-free solution. The quenching by oxygen is easily reversible by removal of dissolved oxygen and completely reversible when all of the oxygen is removed by purging with argon.

The dynamic nature of oxygen quenching (Ru[dpp(SO₃Na)₂]₃)Cl₂ is shown by the FD intensity decays (Fig. 4). This quenching process is believed to be due to energy transfer to form singlet O₂ (5,6,17). In the absence of oxygen, the decay is nearly a single exponential

with a decay time of 3.7 μs . However, a small component with a fractional intensity near 1% and a lifetime of 0.17 μs was observed (Table 1). This short component appears to be unaffected by O_2 in all measurements, which leads us to believe that it may be a result of using the FD method. In fact, previous time domain work shows homogeneous decays with related Ru(II) compounds (18).

The decay times and the intensities in the presence of various amounts of dissolved oxygen were used to determine the oxygen Stern–Volmer quenching constant (Fig. 5). Essentially the same results were obtained from the decrease in intensity and lifetime. The small differences between the various measurements are thought to result from less than complete equilibration with various concentrations of dissolved oxygen. It is important to recognize that the Stern–Volmer quenching constant of $11\,330\text{ M}^{-1}$ means that the probe is highly sensitive to small amounts of dissolved oxygen. A decrease in intensity or lifetime of 10% is readily observable and would occur at an oxygen concentration of 8.8 μM , which is equivalent to equilibration with 5.5 torr of oxygen. With careful measurements of the intensity or lifetimes, still smaller oxygen concentrations could be measured with this water-soluble oxygen probe. This Stern–Volmer quenching constant and unquenched lifetime (3.7 μs) correspond to a bimolecular quenching constant of $3.1 \times 10^9\text{ M}^{-1}\text{ s}^{-1}$, which indicates that the quenching is diffusion controlled.

If the sensor is to be used with biological compounds, it is important to understand whether its intensity, intensity decay or sensitivity to oxygen will be altered by the presence of large quantities of biological substances. Hence, we examined the intensity decay of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ in the presence of DPPG vesicles (Fig. 6) and in the presence of HSA (Fig. 7). The lipid vesicles appear to have minimal effect on the intensity decay of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$. In the absence of oxygen, the intensity decay was essentially the same in the presence or absence of the DPPG vesicles (Table 1). At this time we are not certain whether the modest change in sensitivity to oxygen is in fact due to the interactions of the probe with the lipid vesicles or due to sample-to-sample variations in the measurements. However, the probe does display interactions with HSA (Fig. 7). In the absence of oxygen, the mean decay time increases to 4.1 μs and the decay becomes more heterogeneous. This can be seen by the multiexponential fits in Table 1 and by the increase in the χ^2_{R} values for the single decay time fits. In the presence of HSA the probe also becomes less sensitive to quenching by oxygen. The presence of dissolved oxygen from the air results in decreases in lifetime to 3.1 μs . Hence, the extent of oxygen quenching is clearly sensitive to binding to proteins. However, we note that HSA is an unusual protein with high affinity for hydrophobic anions, and it is not clear that the oxygen sensor will display changes in oxygen sensitivity in the presence of less hydrophobic proteins.

DISCUSSION

What are the potential applications of a water-soluble oxygen sensor? The uses of this sensor in analytical and industrial applications seems quite clear. The compound is chemically stable and can be readily mixed with water. One can imagine tests kits containing dropper bottles with highly concentrated solutions of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)^{2+}$ that can be added to

freshly drawn water samples. The oxygen concentration could then be determined from the intensity or preferably from the decay time of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$. The long decay times of these samples would allow the emission to be detected following the decay of interfering prompt autofluorescence, which can be expected to occur in real samples. One does not frequently encounter long decay time interferences, so that in most cases gated detection and decay time measurements should provide accurate measurements of the oxygen concentration. Another potential application of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ is oxygen imaging in tissues or in cells. In the case of fluorescence microscopy, the $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ could be added to cells by microinjection and the oxygen concentration is determined from the decay times or fluorescence lifetime imaging microscopy (FLIM). One can also imagine alternative structures in which the water-soluble probe is initially made cell permanent by the addition of less polar side chains that are cleaved by intracellular enzymes and then trapped within the cells. We note that $(\text{Ru}[\text{bpy}]_3)\text{Cl}_2$ has been used for oxygen imaging in cells (20) even though its lifetime is 10-fold less than $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)^{2+}$ and thus much less sensitive to oxygen. The present water-soluble probe can be expected to be considerably more sensitive than $(\text{Ru}[\text{bpy}]_3)\text{Cl}_2$.

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Figure 1.
Structure of the water-soluble oxygen sensor.

Figure 2.
Absorption and emission spectrum of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ in water.

Figure 3.
Emission spectra of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ shown at equilibrium with argon, air and 100% oxygen.

Figure 4.
Frequency-domain intensity decays of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ in equilibrium with argon, air or 100% oxygen.

Figure 5.
Oxygen Stern-Volmer plot for (Ru[dpp(SO₂Na)₂]₃)Cl₂ in water at 20°C.

Figure 6. Frequency-domain intensity decays of $(Ru[dpp(SO_3Na)_2]_3)Cl_2$ in the presence of DPPG vesicles (2 mg/mL) at equilibrium with argon and air.

Figure 7. Frequency-domain intensity decays of $(\text{Ru}[\text{dpp}(\text{SO}_3\text{Na})_2]_3)\text{Cl}_2$ in the presence of HSA (5 mg/mL) in phosphate buffer, pH 7.2, at equilibrium with argon and air.

Table 1. Intensity decay analysis of (Ru[dpp(SO₃Na)₂Cl₂]) quenched by dioxygen in various biological media^{*}

Sample	τ_i (μ s)	α_i	f_i	$\langle \tau \rangle$ (μ s) [†]	χ^2_R
H ₂ O, Ar	3.64	0.82	0.99		
	0.166	0.18	0.01	3.61	0.213 (21.3) [‡]
			0.98		
H ₂ O, air	0.953	0.78	0.02		
	0.075	0.22		0.933	1.55 (37.5)
H ₂ O, 100% O ₂	0.227	1.0	1.0	0.227	3.3 (3.3)
HSA, [§] argon	5.44	0.40	0.68		
	2.29	0.41	0.30		
	0.235	0.19	0.02	4.41	0.694 (105)
HSA, [§] air	3.69	0.41	0.78		
	0.733	0.59	0.22	3.03	2.88 (105)
	4.1	0.3	0.68		
	1.12	0.49	0.29		
	0.238	0.2	0.03	3.12	0.566 (244)
DPPG, [§] argon	3.78	0.79	0.99		
	0.171	0.21	0.01	3.73	0.235 (19.8)
DPPG, [§] air	0.734	0.78	0.95		
	0.148	0.22	0.05	0.702	3.01 (23.7)

^{*} Frequency-domain intensity decays obtained with 488 nm excitation and a 610 nm long-pass filter.

[†] Calculated from $\langle \tau \rangle = \sum f_i \tau_i$.

[§] Human serum albumin. (HSA: 5 mg/mL), phosphate buffer, pH 7.2.

[§] Dipalmitoyl-L- α -phosphatidylglycerol (DPPG) lipid vesicles (2 mg/mL), Tris, 50 mM KCl, pH 7.5.

[‡] The values in parentheses are the χ^2_R values for the single decay time fit.